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Self and Non-Self Recognition in a Calcareous Sponge, *Leucandra abratsbo*

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Abstract. Discrimination between self and non-self has been shown in many demosponges, but calcareous sponges have not been studied. Allorecognition in a calcareous sponge, *Leucandra abratsbo*, was analyzed in allogeneic combination assays. Most allogeneic combinations were incompatible, and the low rate (4.8%) of allogeneic acceptances suggests an extensive polymorphism in those genes that may control allorecognition. However, histological studies of the rejection process revealed that the first reaction consisted of strong adhesion of allogeneic pieces. Thereafter, the rejection reaction that followed was accompanied by the accumulation of archeocytes in the contact region. Vigorous cytotoxic reactions occurred within this region, and the degenerated cells were probably phagocytosed by archeocytes, which suggests that they are the primary effector cells for cytotoxicity and phagocytosis. Because *L. abratsbo* is a solitary sponge, armed with protruding spicules that prevent contact of the pinacoderm with that of conspecific individuals, allorecognition may not prevent the formation of allogeneic chimeras in the natural habitat.

Introduction

The immune systems of invertebrates have interested investigators who believe that such systems might be precursors of the vertebrate immune system (Coombe *et al.*, 1984; Stoddart *et al.*, 1985). In the last decade, comprehensive studies have provided much information on sponge allorecognition (Hildemann *et al.*, 1979, 1981; Kaye and Ortiz, 1981; Curtis *et al.*, 1982; Jokiel *et al.*, 1982; Van de Vyver and Barbieux, 1983; Buscema and Van de Vyver, 1984a–c; Neigel and Schmahl, 1984; Neigel and Avise, 1985; Mukai and Shimoda, 1986; Smith and Hildemann, 1984, 1986a, b). The resulting indisputable evidence suggests that allorecognition is the rule in demosponges; alloincompatibility can be induced in most orders of the class Demospongiae.

The allogeneic reactions of demosponges, however, are remarkably variable, so a thorough understanding of sponge allorecognition has been difficult. First, allografts are rejected in some sponges, but accepted in others (Jokiel *et al.*, 1982; Buscema and Van de Vyver, 1984c). Second, the rejection reaction varies considerably from species to species. According to present information, allografts are rejected by cytotoxic reactions (Hildemann *et al.*, 1979, 1981; Buscema and Van de Vyver, 1984b; Mukai and Shimoda, 1986; Smith and Hildemann, 1986a), by the formation of a collagenous barrier (Buscema and Van de Vyver, 1984a, c), or by nonfusion (Buscema and Van de Vyver, 1984c; Mukai and Shimoda, 1986). Moreover, two or three types of rejection reactions have been observed in some species (Van de Vyver and Barbieux, 1983; Buscema and Van de Vyver, 1984c; Mukai and Shimoda, 1986) and the type of allogeneic rejection is independent of sponge phylogeny. Third, various effector cells participate in the rejection reaction. Although several effector cells including archeocytes, collencytes, lophocytes, phagocytes, and amoebocytes have been identified thus far (Van de Vyver and Buscema, 1977; Van de Vyver and Barbieux, 1983; Buscema and Van de Vyver, 1984b, c; Smith and Hildemann, 1986a), we cannot predict which of these cells actually effects rejection reactions (Smith, 1988; Van de Vyver, 1988). Furthermore, we know very little about their origins and transitions as these cells develop normally.

Calcareous sponges diverged from the ancestral sponge before the Devonian period (Hyman, 1940). The shapes and composition of their spicules are distinctly different from those of demosponges, and their allorecognition systems may also be different. In this paper allorecognition in a calcareous sponge (*Leucandra abratsbo*) is presented.

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Most allogeneic combinations were incompatible, suggesting the existence of an extensive polymorphism of histocompatibility genes in natural populations. Vigorous cytotoxic reactions by archeocytes were observed in the contact region. The ecological significance of self and non-self recognition in these sponges is discussed.

Materials and Methods

Sponges

All specimens of *Leucandra abratsbo*, a calcareous sponge with a leuconoid canal system, were collected from a raft at the Breeding Center of Aomori Prefecture in northern Japan. They were abundant in scallop-breeding baskets that were hung a few meters below the water's surface. The size of the raft is about 15 × 20 m, so the maximum distance between any two specimens is about 20 m. The sponge was easily freed from the substratum because it is upright and has a stout body. Once collected, the sponges were put in water-tight containers, brought to the Asamushi Marine Biological Laboratory, and placed immediately in running seawater where they could be maintained for more than ten days. The largest specimen was about 8 cm in length; only those larger than 4 cm were used.

Assessment of incompatibility

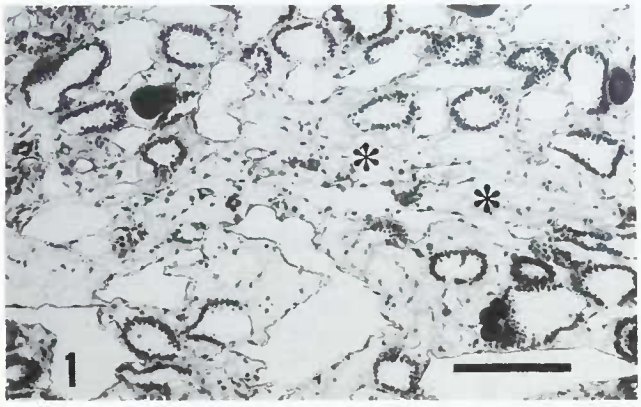
Because parabiosis experiments were not feasible with this sponge, "the allogeneic combination test" was performed as an alternative method. Sponges that had been selected for the allogeneic combination assay, were cut into slices 2 to 3 mm thick, and two sponge pieces derived from different individuals were bound together with a piece of cotton thread. The flatness of their opposed cut surfaces allowed the sponge pieces to be closely appressed, and caused the deeper sponge tissues to be in direct contact. To ensure reliability, the test was first performed with ten replicates of each sponge pair. Because all replicates of a pair showed similar allogeneic reactions, two replicates of each combination were usually performed in this study, unless otherwise mentioned. The polarity of the sponge pieces exerted no influence on their reactions in either allogeneic or autogeneic combinations. Bound sponge pieces were supplied with clean running seawater during the experiments and were as healthy as intact sponges under laboratory conditions. They regenerated the dermal layer and pinacoderm on the free cut surface during the allogeneic combination test.

The bound sponge pieces were examined daily, and most of them were distinctly rejected in four days. In preliminary experiments, five allogeneic combinations that were not rejected in four days did not reject in an additional four days. Thus, all allogeneic combinations that showed no external signs of rejection were fixed with the Bouin's solution five days after binding. To provide a time-series analysis of the rejection process, ten replicates of the same allogeneic combination were constructed from an allogeneic sponge pair. Two of these replicates were fixed daily, embedded in Palaplast, sectioned, and stained with haematoxylin and eosin.

Results

Autogeneic reactions

The fusion process was analyzed morphologically using sponge pieces in autogeneic combinations derived from one sponge specimen. One day after binding, these autogeneic sponge pieces were firmly adherent (Fig. 1). One striking feature in the contact region of such autogeneic combinations is the development of a dermal layer-like tissue between the sponge pieces. Development of this



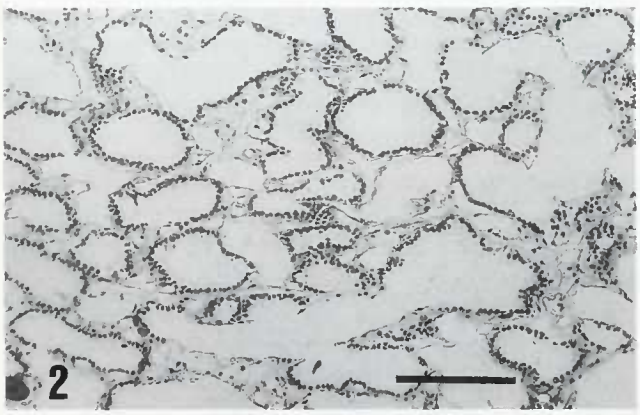
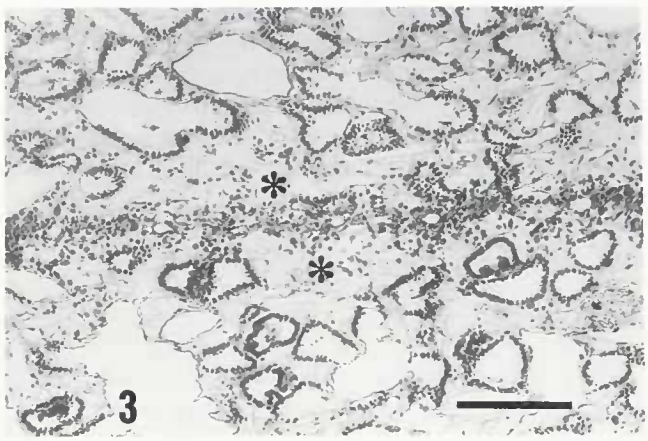


Figure 1. Fusion of a one-day autogeneic combination of *Leucandra abratsbo*. The mid-horizontal line of this photomicrograph is in the interface of the sponge pieces. The dermal layer-like tissue (asterisks) has developed in the contact region. Scale bar = 100 μm.

Figure 2. Fusion of a two-day autogeneic combination of *L. abratsbo*. Mid-horizontal line is in the interface, however, choanocyte chambers are arranged almost regularly. No dermal layer-like tissue is observable. Scale bar = 100 μm.



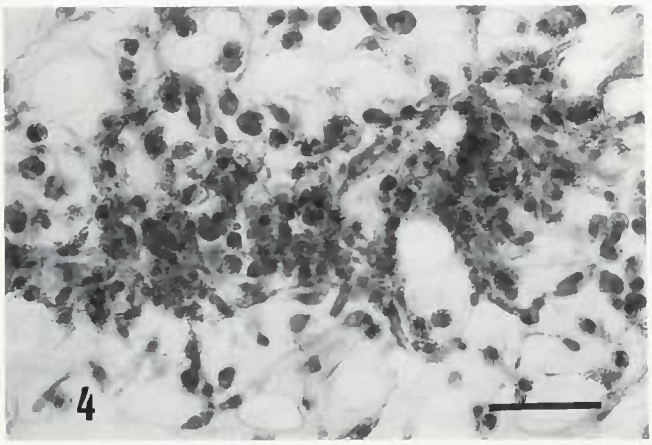


Figure 3. Rejection reaction in a one-day allogeneic combination of *Leucandra abratsbo*. Although these sponge pieces look like fusion from external observation, many archeocytes have already gathered in the contact region. The dermal layer-like tissue (asterisks) is observable on the both sides of the archeocyte accumulation. Scale bar = 100 μm.

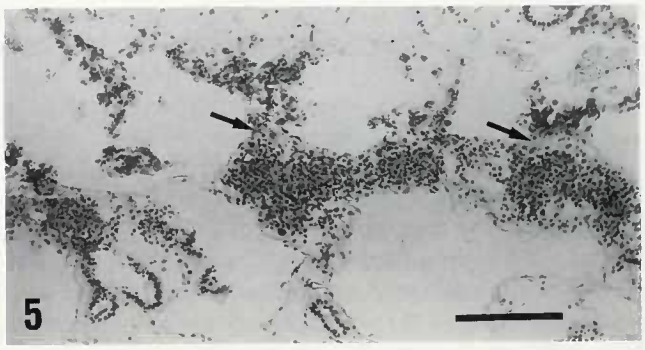
Figure 4. Archeocytes accumulated in the contact region of a one-day allogeneic combination. These archeocytes are in contact with each other, and most of them already show nuclear condensation. Scale bar = 50 μm.

tissue seems to be necessary for the fusion process, facilitating the adhesion of the sponge pieces during the early stages. Only a few archeocytes were found within the contact region.

Two days after binding, the autogeneic sponge pieces were more intimately fused than on day one, so that their external boundaries became obscure. Figure 2 shows the contact region of such sponge pieces. The dermal layer-like tissue has disappeared, and the choanocyte chambers are arranged almost regularly, with no evidence of cytotoxic or phagocytic reactions. The autogeneic sponge pieces fused rapidly, and after four days, the interface between the sponge pieces was almost undetectable microscopically; this signaled that the fusion process was complete.

Allogeneic reactions

The rejection process occurring in allogeneic combinations was studied histologically using daily samples from sets of coupled sponge pieces, each set derived from two physiologically discrete individuals of the same species. Four incompatible sponge pairs were thus observed, and they all showed a similar rejection process. The rejection process of only one allogeneic sponge pair is represented. One day after binding, allogeneic sponge pieces had adhered firmly and their pinacoderms were already fused. They are therefore difficult to distinguish from autogeneic combinations by external observation. Nevertheless, the rejection process has already begun microscopically. Figure 3 shows the contact region of a one-day allogeneic combination. As in autogeneic fusions, dermal layer-like tissue has developed in the contact region, and the sponge pieces are firmly adhered. In contrast to autogeneic fusion, archeocyte accumulations are already visible in the contact region (Fig. 4, an enlargement). The archeocytes are congregated and in close contact with each other. Within this



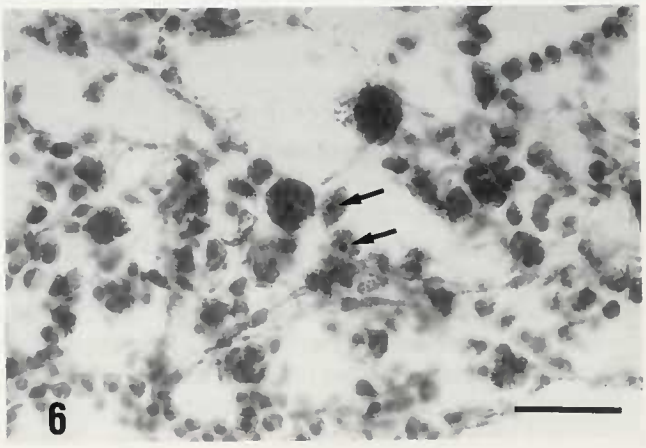
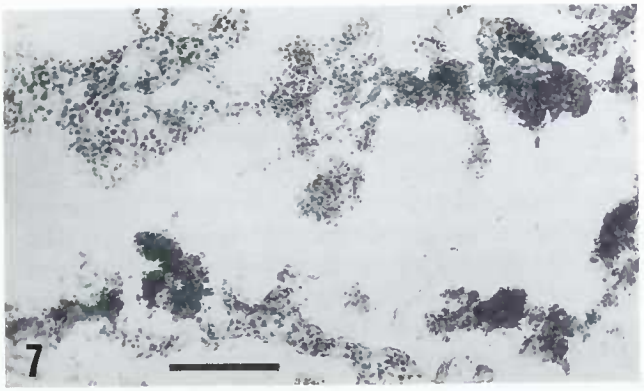


Figure 5. Rejection reaction in a two-day allogeneic combination of *Leucandra abratsbo*. Extensive degeneration of the archeocyte accumulation is shown. These sponge pieces are splitting off (arrows). Scale bar = 100 μm.

Figure 6. Phagocytes and archeocytes with conspicuous nuclear condensation (arrows) in a two-day allogeneic combination. Scale bar = 50 μm.



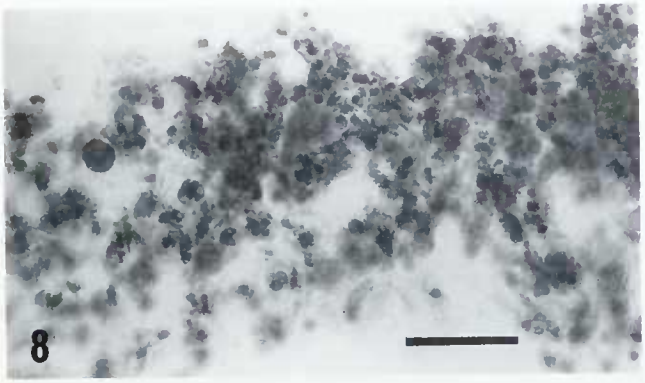
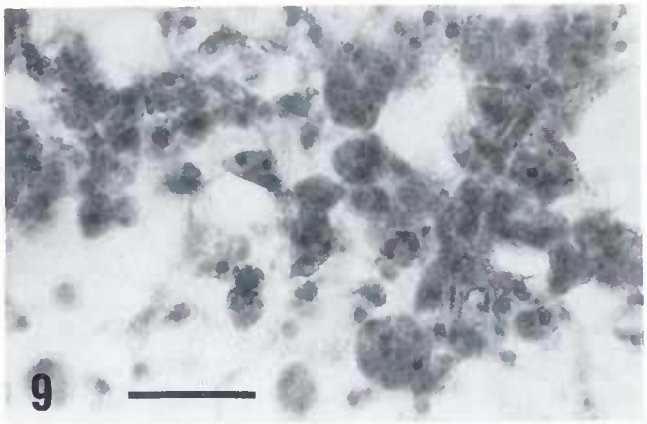


Figure 7. Rejection reaction in a four-day allogeneic combination of *Leucandra abratsbo*. The aggregates of the degenerated archeocytes have split off. Scale bar = 100 μm.

Figure 8. A large aggregate of degenerated cells in a four-day allogeneic combination. Scale bar = 50 μm.



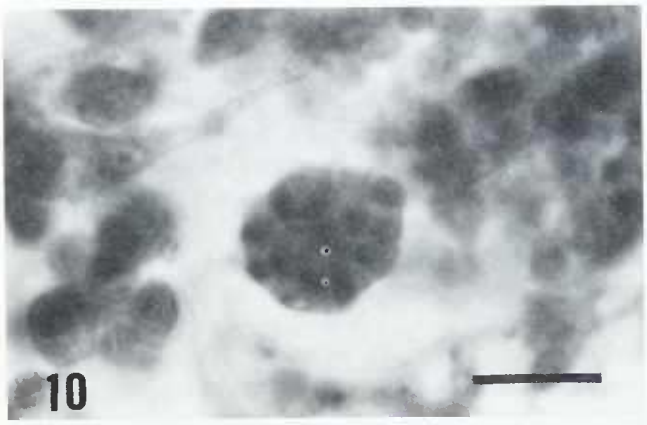


Figure 9. Many phagocytes and degenerated cells in a four-day allogeneic combination. Scale bar = 50 μm.

Figure 10. A large phagocyte that has engulfed more than ten degenerated cells. Scale bar = 10 μm.

cell accumulation, cytotoxic reactions are evident, because numerous cells show degenerative nuclear condensation. The cytotoxic reaction mediated by the archeocytes apparently begins soon after they accumulate and make cell contact.

Two days after the allogeneic sponge pieces had been bound, external signs of rejection are already evident. The fused pinacoderm begins to break along the boundary between the sponge pieces. Figure 5 shows massive accumulations of archeocytes in the contact region. Within this cell accumulation, tissue degeneration is obvious, and the sponge pieces begin to split off (Fig. 6, enlargement). Nuclear condensation is clearly visible in the degenerated cells. Phagocytosis is already discernible, and phagocytes that had engulfed several degenerated cells can be seen.

Four days after binding, extensive necrotic tissue (about 0.5 mm thick) is visible between allogeneic sponge pieces. Because it has become frail, the sponge pieces fall apart if the binding thread is removed (Fig. 7). The necrosis is, however, limited to the contact region, and the sponge tissues external to it show no degenerative signs. Thus, archeocytes, at least at four days, had apparently not invaded far into the allogeneic tissues. In the necrotic cell masses, nuclear condensation is evident in most of the cells, and cell lysis prevails (Fig. 8). The numbers of phagocytotic figures have increased considerably, and Figure 9 shows such phagocytosis in the contact region. Figure 10 reveals a phagocyte that has engulfed more than ten

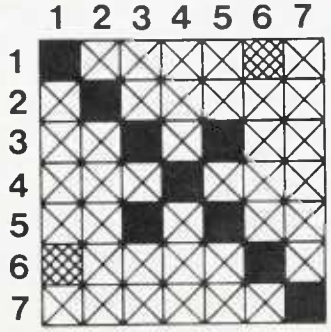


Figure 11. Reactions of allogeneic and autogeneic combinations between the seven individuals of *Leucandra abratsbo*. ☒, allogeneic rejection; ▩, weak rejection; ■, autogeneic fusion or allogeneic acceptance.

Table I

Allogeneic reactions in Leucandra abratsbo

	Number of combinations scored	%
Acceptance	6	4.8
Weak rejection	9	7.1
Rejection	112	88.2
Total	127	100.1

degenerated cells. Later, these sponge pieces were completely disjoined, and regeneration of pinacoderm on the re-exposed surface was observed.

Patterns and frequencies of allorecognition

Figure 11 represents an example of autogeneic and allogeneic combinations among seven individuals. All the combinations were tested twice and gave similar results. One acceptance and one weak rejection was found among the allogeneic combinations, but all of the other allogeneic combinations rejected vigorously. Seven autogeneic combinations fused completely.

Table I shows the cumulated results of 127 allogeneic combinations. About 5% of the combinations were accepted, while the others were incompatible. Histological examination of the allogencic acceptances revealed no rejection reactions; neither archeocyte accumulation nor cytotoxic reactions were observed at the interface of the accepted sponge pieces. Accordingly, they were indistinguishable from autogeneic fusions. In about 7% of the allogeneic combinations, the sponge pieces rejected, but weakly. These weak rejections were hardly distinguishable from the allogeneic acceptances by external observation, because the fused pinacoderm was not broken. Nevertheless, histological examination revealed distinct rejection reactions in the contact region.

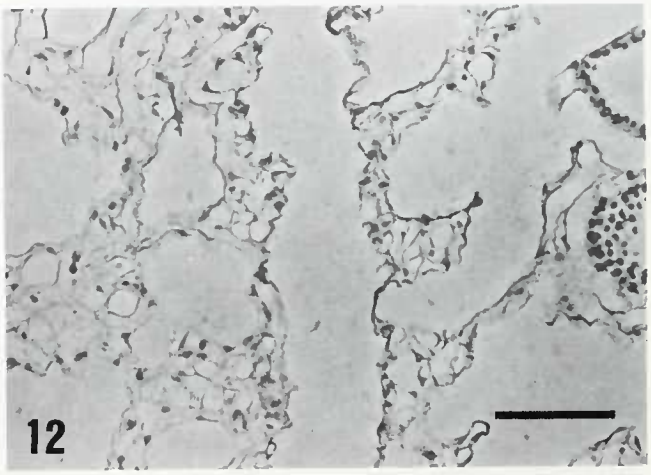
Parabiosis experiments

Parabiosis experiments, in which two sponge pieces are touching surface-to-surface, were tried. Figure 12 shows the result of an autogeneic parabiosis, and Figure 13 shows an allogeneic combination, both five days after binding. Both figures show a wide gap between the sponge surfaces because the densely protruding spicules, which have been dissolved in the Bouin's solution, prevented contact between the opposing pinacoderms. Obviously, no fusion or rejection reaction has occurred in these sponge pieces.

Discussion

Alloincompatibility in a calcareous sponge, *Leucandra abratsbo*, is shown for the first time in this report. These results suggest an extensive polymorphism of histocompatibility genes, because most individuals are alloincompatible. Obviously, further studies on other calcareous sponges are necessary to determine whether allorecognition specificity is a general phenomenon in the class Calcarea.

In this calcareous sponge, allogeneic pieces were rejected by cytotoxic reactions. Neither collagen deposition (chronic rejection) nor nonfusion was observed, although they have been shown in demosponges (Buscema and Van de Vyver, 1984a–c; Mukai and Shimoda, 1986). Many archeocytes accumulated in the contact region of allogeneic combinations of this calcareous sponge; mesohyl cell accumulations have been observed in many demosponges (reviewed by Smith, 1988). Direct contact between



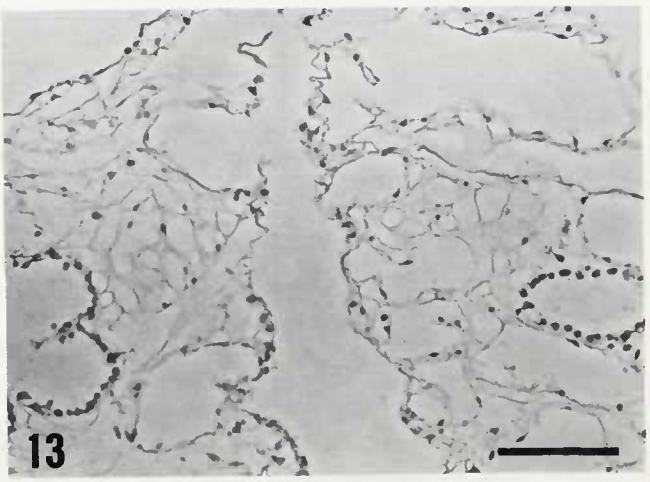


Figure 12. A parabiosis experiment of autogeneic sponge pieces five days after binding. They have not fused, and there is a wide space between their opposing pinacoderms. Calcareous spicules that prevented contact of the sponge surfaces have been dissolved in the Bouin's solution. Scale bar = 25 μm.

Figure 13. A parabiosis experiment of allogeneic sponge pieces five days after binding. There is no sign of a rejection reaction. Scale bar = 25 μm.

archeocytes is most probably necessary for the cytotoxic reaction to be triggered, because it occurred only within cell accumulations in which archeocytes were in close contact with each other. In demosponges, the necessity for contact between mesohyl cells has been suggested (Bigger *et al.*, 1981), but the involvement of diffusible substances is also plausible (Smith and Hildemann, 1986a, b). Until now, there has been no evidence that archeocytes selectively come into contact with allogeneic cells. To answer this question, *in vitro* studies may be helpful. In a solitary ascidian, *Halocynthia roretzi*, Fuke (1980) showed that the cytotoxic reaction between allogeneic coelomocytes *in vitro*, termed the "contact reaction," occurs after close contact between allogeneic cells.

Bound sponge pieces of *L. abratsbo* adhered firmly within 24 h in allogeneic combinations, as well as in autogeneic ones. By this time, the dermal layer-like tissue has developed in the contact region, and this intervening tissue may play an important role in the adhesion of sponge pieces. Because this dermal layer-like tissue formed similarly in allogeneic and autogeneic combinations, its formation is not an allogeneic reaction but more likely a regenerative event induced by the exposure of inner tissues to the exterior. Indeed, the composition of the dermal layer-like tissue was similar to that of the dermal layer that regenerated on the reverse side of the sponge pieces. Pinacoderm was formed on the surface of the dermal layer-like tissue after the sponge pieces were disunited. In autogeneic fusions, however, it disappeared from the contact region within a few days. Therefore, the dermal layer-like tissue is conceivably a regenerated dermal layer in the contact region.

About 95% of the allogeneic combinations of *L. abratsbo* were incompatible in natural populations collected from a raft (15 × 20 m). This high rate of alloincompatibility reflects extensive dispersion of sponge larvae. This calcareous sponge released amphiblastula larvae in the morning, and they swam actively, settled, and metamorphosed on the substratum (Amano, in prep.). Before settlement, they crawled about on the substratum for several hours. In demosponges, also, larval release is controlled by light (Amano, 1986, 1988); phototaxis and geotaxis enable the swimming larvae to settle in a suitable site, often at a considerable distance (Bergquist *et al.*, 1970). Twenty-four hours of swimming and transport by water currents are probably sufficient for the released amphiblastula larvae to be dispersed beyond the limits of the raft (15 × 20 m). Therefore, some specimens used in this study may be kin, and allogeneic combinations of the sponges with kinship may result in fusion. Not knowing the genealogies of the tested specimens, however, we cannot know whether allogeneic acceptances necessarily imply genetic identity of the combined individuals of *L. abratsbo* (Grosberg, 1988).

Because *L. abratsbo* is densely covered with protruding stout spicules, they prevented sponge pieces from touching each other when parabiosis experiments were tried. Without immediate contact between their opposing pinacoderms, the sponge pieces did not fuse, nor were there rejection reactions even in autogeneic or allogeneic individuals. Therefore, allorecognition in this sponge is not required to avoid fusion and the formation of allogeneic chimeras in nature. But if it is so, why has this sponge developed a recognition system that can be revealed only in the laboratory? Grosberg (1988) has discussed the evolution and ecological significance of allorecognition systems in clonal invertebrate-organisms that have numerous opportunities for tissue contacts between isogeneic and allogeneic individuals. In solitary invertebrates, however, conspecific interactions rarely occur during the life cycle. Accordingly, we cannot assume that allorecognition specificity is the only phenotypic effect of genes controlling allorecognition, particularly in solitary invertebrates (Grosberg, 1988, 1989; Grosberg and Quinn, 1988). This study indicates that *L. abratsbo*, a solitary sponge, has few opportunities for tissue contacts in nature. Thus, allorecognition specificity may be an epiphenomenon resulting from pleiotropic genes. Although the ecological significance of allorecognition specificity is as yet unknown in invertebrates, pleiotropic models have been proposed and supported experimentally; *e.g.*, the control of gametic incompatibility (Oka, 1970; Scofield *et al.*, 1982; Fuke, 1983), and the discrimination of food bacteria (Wilkinson, 1984; Wilkinson *et al.*, 1984). In conclusion, this study supports the idea that self and non-self recognition is a general phenomenon in the lowest metazoan phylum, the sponges.

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